IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: HADLACZKY *et al.* Customer No.: 24961

Serial No.: 09/724,726 Group Art Unit: 1638

Filed: November 28, 2000 Examiner: Helmer, G.L.

For: ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

I, Steven F. Fabijanski, declare as follows:

1) I am familiar with the subject matter of the above-captioned application, which was filed on November 28, 2000.

2) I received a Bachelor's degree in Biology from the University of Miami (Florida) in 1977. I received a Ph.D. degree in Cellular and Molecular Biology from the University of Southern California in 1981. I have held post-doctoral positions at the University of Ottawa in Ottawa, Ontario, Canada and the University of Southern California in Los Angeles, California from 1982 to 1985. From 1986 to 1991, I held the position of Research Director at Paladin Hybrids, Inc.

3) I have over 20 years of experience in the areas of plant molecular biology, plant gene expression, plant tissue and cell culture and development of techniques to produce genetically modified plants and plant artificial chromosomes. I have authored or co-authored over 20 publications and I am an inventor of 15 US and foreign patents.

4) I am currently Research Director at Agrisoma Biosciences Inc., located in Saskatoon, Saskatchewan, Canada. Chromos Molecular Systems, Inc., located at 8081 Lougheed Highway, Burnaby, B.C., Canada V5A 1W9, is an owner of Agrisoma Biosciences, Inc. I have held this position since 2001. I am also President of FAAR Biotechnology Group Inc., located at Suite 323, 5929L Jeanne D'arc Boulevard, Orleans, Ontario, Canada K1C 7K2. I have held this position since 1992.

5) In my capacity as researcher, myself, persons under my direction and other research groups: the Scottish Crop Research Institute in Scotland; the Danforth Plant Science Center in St. Louis, Missouri; the Hungarian Biological Research Center in Hungary; and Applicant's research group at the Plant Biotechnology Institute in Saskatoon, Saskatchewan, Canada; have studied the introduction of satellite artificial chromosomes into plant cells. Using methods and materials described in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have demonstrated that satellite artificial chromosomes can be transferred to plant protoplasts using (1) microcell-mediated fusion of satellite artificial chromosome-containing murine cells with plant protoplasts; or (2) lipid-mediated transfection of isolated satellite artificial chromosomes into plant protoplasts.

As exemplified by the results shown below, we have demonstrated element-for-element and step-for-step that, by following the teachings in the application, one can (i) transfer satellite artificial chromosomes into plant cells; and (ii) detect satellite artificial chromosome material in the plant cells beginning at about 24 h post-transfer to about at least 16 weeks following transfer of the satellite artificial chromosomes into the plant cells.

I. Materials and Methods

Transfer of satellite artificial chromosomes into Tobacco Cells

Satellite artificial chromosomes were introduced into Tobacco cells using microcell-mediated fusion. Microcells were prepared from murine cells containing a satellite artificial chromosome as described in U.S. Patent No. 6,077,697 (of record in an Information Disclosure Statement filed May 14, 2001, in connection with the above-captioned application). Briefly, 5×10^6 EC3/7C5 cells (a mouse cell line deposited by Applicant in accord with the Budapest Treaty at the European Collection of Animal cell Culture (ECACC)

under Accession No. 96040925) in a T25 flask were treated first with 0.05 μg/ml colcemid for 48 hr and then with 10 μg/ml cytochalasin B for 30 min. The T25 flask was centrifuged on edge and the pelleted microcells were suspended in serum free DME medium. The microcells were filtered through first a 5 micron and then a 3 micron polycarbonate filter, treated with 50 μg/ml of phytohemagglutin, and used for polyethylene glycol mediated fusion with recipient cells.

The microcells containing satellite artificial chromosomes were then fused with freshly prepared tobacco BY-2 protoplasts in a ratio of 10:1 (microcells to protoplasts). Fusion occurred in the presence of 20% PEG 4000 and 100-200 mM calcium chloride.

4',6-diamidino-2-phenylindole.2HCl (DAPI) staining of the microcells by preincubation of the microcells with DAPI at a final concentration of 1 μg/ml allowed visualization of the fusion and transfer of the chromosomes to the tobacco protoplasts. The fused protoplasts were recovered and allowed to grow for one or more generations.

To further demonstrate the transfer of mouse chromosomal sequences to tobacco protoplasts, the fused tobacco cell nuclei were isolated and subjected to fluorescence *in situ* hybridization (FISH) analysis using biotin-labelled mouse major satellite DNA as a probe. To isolate nuclei from the fused tobacco cells, protoplast calli were digested with 1.2% Cellulase 'Onozuka' R-10 and 0.4% w/v Macerozyme R-10 in nuclei isolation buffer (10 mM MES-pH 5.5, 0.2M sucrose, 2.5 mM EDTA, 2.5 mM DTT, 0.1 mM spermine, 10 mM NaCl, 10 mM KCl and 0.15% Triton X-100) for 3 hours. After centrifugation at 80 x g for 10 minutes, the pellets of protoplasts were resuspended in hypertonic buffer of 12.5% W5 solution (Hinnisdaels *et al.* (1994) *Plant Molecular Biology Manual G2*:1-13, Kluwer Academic Publisher, Belgium) for 10 minutes. To promote disruption of protoplasts, the protoplast suspension was forced through a syringe needle four times. The disrupted protoplasts were filtered through 5

micron meshes to remove debris and centrifuged at 200 x g for 10 min. By repeated washing of the pellet in a nuclei isolation buffer containing phenylmethylsulfonylfluoride (PMSF) and centrifugation at 200 x g for 10 minutes, nuclei were collected as a white pellet freed from cytoplasm contamination and cellular debris. Samples were fixed in 3:1 methanol:glacial acetic acid for FISH analysis.

FISH analysis to screen for the transfer of artificial chromosomes into plant cells was performed using DNA probes specific for the mouse major satellite DNA as described (Fransz *et al.*, *The Plant Journal*, 9: 421-430, 1996; being provided in an Information Disclosure Statement filed on the same day herewith).

Transfer of satellite artificial chromosomes into *Arabidopsis* Cells

Satellite artificial chromosomes were introduced into *Arabidopsis* cells using microcell-mediated fusion. Microcells were prepared as described above. The prepared microcells were then fused with freshly prepared *Arabidopsis* protoplasts in a ratio of 10:1 (microcells to protoplasts). Fusion occurred in the presence of 25% PEG 6000, 204 mM $CaCl_2$, pH 6.9 within the first 5 minutes of mixing. Typically, less than about one minute of mixing is required to observe fusion between microcells and protoplasts. Fused cells were washed with 240 mM $CaCl_2$, then floated on top of a solution of 204mM sucrose in B5 salts. Cells were then transferred to cell suspension culture media (MS, 87mM sucrose, 2.7 μM napthalene acetic acid, 0.23 μM kinetin, pH 5.8).

Fused protoplasts were recovered and allowed to grow for one or more generations. Southern hybridization and PCR analysis using satellite sequences known to exist on the satellite artificial chromosome, were used to detect the presence of satellite artificial chromosomes in the fused protoplasts. To further demonstrate the transfer of mouse chromosomal sequence to *Arabidopsis* protoplasts, *Arabidopsis* plant cell nuclei were isolated and subjected to FISH analysis using biotin-labelled mouse major satellite DNA as a probe, as described

above.

Transfer of satellite artificial chromosomes into Rice Protoplasts

Isolated murine artificial chromosomes (MACs) prepared by sorting through a FACS apparatus and purified as described, *e.g.*, in U.S. Patent No. 6,077,697 (of record as described above), were transferred into rice plant protoplasts by cationic lipid- mediated transfection. First, 1 x 10^6 purified artificial chromosomes in liquid buffer were mixed with 15μl of LipofectAMINE 2000 (Gibco, Md, USA). The satellite artificial chromosome/LipofectAMINE solution was allowed to complex for three hours, and then was added to a mixture of 1 X 10^5 freshly prepared rice protoplasts.

The uptake of the lipid-complexed artificial chromosome was monitored by adding to the mixture of protoplasts and purified artificial chromosomes a fluorescent dye that stains DNA. Microscopic examination of the protoplast/artificial chromosome mixture over the next several hours allowed the visualization of the artificial chromosome being transported across the protoplast cellular membrane and the presence of the readily identifiable MAC in the cytoplasm of the rice plant cell.

II. Results

The results provide evidence of satellite artificial chromosome delivery to plant cells by microcell-mediated fusion or lipid-mediated transfection. The results further demonstrate that transferred satellite artificial chromosome materials are detected in plant cells for at least 16 weeks following such transfer.

In tobacco cells, protoplast fusion and artificial chromosome transfer were confirmed by DAPI staining. In addition, FISH analysis of nuclei isolated from tobacco protoplasts fused with satellite artificial chromosome-containing murine micro cells showed numerous nuclei that had incorporated satellite artificial chromosome material.

In *Arabidopsis* cells, Southern hybridization and PCR analysis demonstrated the presence of satellite artificial chromosome-related sequences in fused *Arabidopsis* protoplasts. In addition, FISH analysis demonstrated that a portion of the nuclei isolated following protoplast fusion contained mouse major satellite DNA. These results indicated successful transfer of one or more mouse chromosomes to the *Arabidopsis* nuclei. Further, PCR data indicated that satellite artificial chromosome material was still present in *Arabidopsis* protoplasts after 8 weeks of culture. In addition, FISH analysis undertaken on samples from the same *Arabidopsis* cultures 16 weeks post-fusion demonstrated the presence of satellite artificial chromosome material.

In rice protoplasts, isolated mammalian satellite artificial chromosomes were delivered by lipid-mediated transfection. Fluorescent dye analysis demonstrated incorporation of the purified artificial chromosome into rice protoplasts.

In all cases, microscopic evidence of the uptake of satellite artificial chromosomes into plant cells and FISH analysis of plant cell nuclei revealed the presence of satellite artificial chromosome material in the plant cells within 24 hours of culture, and at rates of 1-5%

III. Conclusion

The above experiments demonstrate that satellite artificial chromosomes can be transferred to plant cells. Evidence of satellite artificial chromosome transfer to plant cells can be detected within 24 h and for up to at least 16 weeks following such transfer. Thus, by following the teachings of the specification and standard methods as described herein, one can introduce satellite artificial chromosomes into plant cells.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Steven F. Fabijanski

Date July 16, 2003